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16. ABSTRACT

In vitro methods of assessing alterations in drug metabolism and the measurement of lindane metabolites in urine were compared for their ability to determine the influence of ethanol on drug metabolism. Ethanol was administered ip to young adult female rats daily for seven days at doses of 0.12, 0.60 and 3.0 ml/kg. No alterations were observed in ethylmorphine demethylation, hexobarbital oxidation or glucuronyltransferase. Aniline hydroxylation was decreased at the high dose level and aldrin epoxidation was increased at the intermediate dose. In vivo only the high dose of ethanol produced significant changes with significant increases observed for the oxidation of lindane to alcohol metabolites, the glucuronidation of the alcohol but not the chlorophenol metabolites, and glutathione conjugation. The latter increase was also observed in vitro. The in vivo and in vitro data suggest a minimal effect of ethanol on drug metabolism at low levels of administration.

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COMPARISON OF IN VITRO METHODS AND THE IN VIVO METABOLISM OF LINDANE FOR ASSESSING THE EFFECTS OF REPEATED ADMINISTRATION OF ETHANOL ON HEPATIC DRUG METABOLISM

(Liver; conjugation reactions; mixed function oxidases)

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SUMMARY

In vitro methods of assessing alterations in drug metabolism and the measurement of lindane metabolites in urine were compared for their ability to determine the influence of ethanol on drug metabolism. Ethanol was administered intraperitoneally (i.p.) to young adult female rats daily for 7 days at doses of 0.12, 0.60 and 3.0 ml/kg. No alterations were observed in ethylmorphine demethylation, hexobarbital oxidation or glucuronyltransferase. Aniline hydroxylation was decreased at the high dose level and aldrin epoxidation was increased at the intermediate dose. In vivo only the high dose of ethanol produced significant changes with significant increases observed for the oxidation of lindane to alcohol metabolites, the glucuronidation of the alcohol but not the chlorophenol metabolites, and glutathione conjugation. The latter increase was also observed in vitro. The in vivo and in vitro data suggest a minimal effect of ethanol on drug metabolism at low levels of administration.

INTRODUCTION

The effects of ethanol on the liver, either given as a single dose or following prolonged administration, have been well documented. Its influence on drug metabolism has been studied by a number of investigators, but discrepancies appear

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in the literature, some of which are due to differences in protocols. Chronic treatment has been reported to result in proliferation of the smooth endoplasmic reticulum and an increase in drug metabolism [1–3]. Ionnides et al. [4] found that i.p. administration of ethanol to male rats at doses as low as 10 mg/kg/day for 3 days caused increases in biphenyl-4-hydroxylase and NADPH cytochrome *c* reductase. Ethylmorphine demethylase and cytochrome P-450 content were not altered. Valerino et al. [5] reported that i.p. doses of 4 g/kg/day for 7 days but not 2 g/kg/day decreased aniline hydroxylase in male rats. Although ethylmorphine demethylation was not altered by either dose, cytochrome P-450 content was increased by the lower dose and decreased by the higher dose. Conjugation reactions have also been studied. Ethanol, 6 g/kg for 2 days followed by 12–15 g/kg/day for 3 weeks given in the diet of female rats, increased glutathione-*S*-transferase activity with both 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane as substrates [6].

The effects of alcohol on drug metabolism in vivo have been less well studied. Schuppel and Kuthe [7] reported that 3 ml/kg ethanol given by gavage to male and female rats did not alter hexobarbital elimination from blood. The purpose of the present study was to treat rats with ethanol and compare commonly used in vitro measurements of drug metabolism with the in vivo system of Chadwick [8,9], in which lindane serves as a model substrate, for their abilities, both quantitatively and qualitatively, to detect the influence of ethanol on both phase I and II reactions associated with xenobiotic metabolism.

MATERIALS AND METHODS

In both the studies made on in vitro measurements and those on lindane metabolism in vivo, young female Fischer 344 rats (Charles River Breeding Laboratories, Kingston, NY) were used. They were housed singly in stainless steel cages with free access to food (Purina Lab Chow, Ralston Purina Co., St. Louis, MO) and water. Lights were on a 12:12 h light:dark cycle with the temperature maintained at 21°C. Bedding was Bed-O-Cobs (Andersons Cob Division, Delphi, IN).

After an adaptation period of one week, ethanol (U.S. Industrial Chemicals Co., New York, NY) was administered i.p. to groups of 6 rats at doses of 0.12, 0.6 and 3 ml/kg daily for 7 days. The dosing volume was 1% of body weight. 24 h After the last dose, blood was obtained from the orbital sinus for measurement of plasma sorbitol dehydrogenase [10]. Whole liver homogenates were used for determination of glucose-6-phosphatase [11] and the glucuronidation of 1-naphthol and chloramphenicol [12]. Microsomal and cytosolic fractions were prepared by differential centrifugation. Ethylmorphine demethylation was measured by the method of LaDu et al. [13], aniline hydroxylation by the method of Imai et al. [14], hexobarbital oxidation by the method of Kupfer and Rosenfeld [15,16] and aldrin epoxidation by the

method of Wolff et al. [17]. Glutathione-*S*-transferase activity in the cytosolic fraction was determined using the aryl substrate 1,2-dichloro-4-nitrobenzene and the epoxide substrate 1,2-epoxy-3-(*p*-nitrophenoxy)propane [18]. Proteins were quantified using the method of Lowry et al. [19].

On day 8 all rats in the in vivo study were dosed subcutaneously (s.c.) with 20 mg lindane (containing 3.5 μCi [U-^{14}C] lindane)/kg. [U-^{14}C] lindane, with a specific activity of 54 mCi/mmol and a radiochemical purity of 98%, was obtained from Amersham (Arlington Heights, IL). Following treatment on day 8 the animals were transferred to animal containment chambers (Plas-Labs, Lansing, MI) and urine, feces and expired air were collected for 24 h.

A 10-ml urine aliquot was made acid (pH 2.0) with HCl and extracted twice with equal volumes of isooctane for free chlorophenols, alcohols and unaltered lindane. The residual urine was hydrolyzed with β-glucuronidase (Sigma, St. Louis, MO) by a previously reported procedure [20] and, after acidification, was extracted twice with equal volumes of isooctane to obtain the chlorophenol and alcohol sulfates. Residual urine from this extraction was hydrolyzed and acylated as previously described by Allsup and Walsh [21] and extracted twice with isooctane for determination of the mercapturic acid metabolites of lindane. Urine extracts were analyzed for lindane metabolites on a Tracor Model MT-220 gas chromatograph equipped with ^{63}Ni electron-capture detector and linearizer. The column consisted of a 183 × 0.63 cm O.D. glass U-tube packed with 5% EGS + 1% H_3PO_4 on 90/100-mesh Anakrom A (Analabs, North Haven, CT). The column was maintained isothermally at 170°C. Inlet and detector temperatures were 245°C and 330°C, respectively. The 5% methane/argon carrier gas flow rate was regulated at 55 cm^3/min. Radioactivity was analyzed in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer using a quench correction curve and a scintillation solution containing 500 ml toluene, 500 ml cellosolve, 5.0 g 2,5-diphenyloxazole (PPO) and 50 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP).

Duncan's Multiple Range Test [22] was used as an aid in the interpretation of the data from this study. Treatments were considered significantly different at $P < 0.05$.

RESULTS

The doses used in the present study were slightly hepatotoxic (Table I). The intermediate dose of 0.60 ml/kg caused a slight decrease in hepatic glucose-6-phosphatase activity. The higher dose of 3.00 ml/kg caused an increase, suggesting perhaps an effect on membrane fluidity. The increases in serum sorbitol dehydrogenase were minimal. Attempts to raise the daily exposure levels resulted in excessive mortality (data not shown). In agreement with other investigators, ethylmorphine demethylation and hexobarbital oxidation were not altered [4,5,7], but aniline hydroxylation was decreased at the high dose level (Table II). Aldrin epoxidation was increased only by the intermediate dose level (0.60 ml/kg).

TABLE I

EFFECT OF ETHANOL ON INDICATORS OF HEPATOTOXICITY

Treatment[a]	Glucose-6-phosphatase (μmol/g liver/min)	Sorbitol dehydrogenase (μmol fructose/ml serum/h)
Control	15.5 ± 0.4[b]	0.98 ± 0.10[b]
Ethanol, 0.12 ml/kg	15.5 ± 0.7[b]	1.39 ± 0.06[c]
Ethanol, 0.60 ml/kg	12.6 ± 0.2[c]	1.36 ± 0.07[c]
Ethanol, 3.00 ml/kg	21.2 ± 0.5[d]	1.41 ± 0.05[c]

[a]Ethanol was administered i.p. to groups of 6 rats for 7 days. Rats received 1 ml/100 g body wt. Controls received distilled water. Rats were killed 24 h after the last dose.
[b,c,d]Values are means ± S.E.M. Values within the same column with different superscripts are significantly different ($P \leq 0.05$).

TABLE II

EFFECT OF ETHANOL ON MICROSOMAL XENOBIOTIC METABOLISM MEASURED IN VITRO

Treatment[a]	Ethylmorphine demethylation (nmol HCHO/mg protein/min)	Hexobarbital oxidation (nmol/mg protein/min)	Aniline hydroxylation (nmol/mg protein/min)	Aldrin epoxidation (nmol/mg protein/min)
Control	2.62 ± 0.09[b]	2.39 ± 0.23[b]	0.68 ± 0.06[b,c]	0.309 ± 0.035[b]
Ethanol, 0.12 ml/kg	3.21 ± 0.09[b]	2.77 ± 0.19[b]	0.54 ± 0.05[b]	0.316 ± 0.026[b]
Ethanol, 0.60 ml/kg	2.98 ± 0.13[b]	2.74 ± 0.38[b]	0.80 ± 0.06[c]	0.520 ± 0.024[c]
Ethanol, 3.00 ml/kg	3.41 ± 0.48[b]	2.66 ± 0.29[b]	0.34 ± 0.02[d]	0.314 ± 0.025[b]

[a]Ethanol was administered i.p. to groups of 6 rats for 7 days. Rats received 1 ml/100 g body wt. Controls received distilled water. Rats were killed 24 h after the last dose.
[b,c,d]Values are means ± S.E.M. Values within the same column with different superscripts are significantly different ($P \leq 0.05$).

TABLE III

EFFECT OF ETHANOL ON CONJUGATION REACTIONS

Treatment[a]	Glutathione-*S*-transferases		Glucuronyltransferases	
	1,2-Dichloro-4-nitrobenzene (nmol/mg protein/min)	1,2-Epoxy-3-(*p*-nitrophenoxy)-propane (nmol/mg protein/min)	1-Naphthol (nmol/g liver/min)	Chloramphenicol (nmol/g liver/min)
Control	20.0 ± 0.7[b]	53.5 ± 3.5[b]	1378 ± 210[b]	26.0 ± 2.4[b]
Ethanol, 0.12 ml/kg	23.8 ± 1.1[c,d]	69.3 ± 6.2[c]	1146 ± 94[b]	21.2 ± 1.4[b]
Ethanol, 0.60 ml/kg	26.0 ± 0.5[c]	64.6 ± 4.1[b,c]	1394 ± 88[b]	22.6 ± 2.5[b]
Ethanol, 3.00 ml/kg	22.9 ± 1.1[d]	63.1 ± 3.1[b,c]	1506 ± 226[b]	22.5 ± 1.3[b]

[a]Ethanol was administered i.p. to groups of 6 rats for 7 days. Rats received 1 ml/100 g body wt. Controls received distilled water. Rats were killed 24 h after the last dose.
[b,c,d]Values are means ± S.E.M. Values within the same column with different superscripts are significantly different ($P \leq 0.05$).

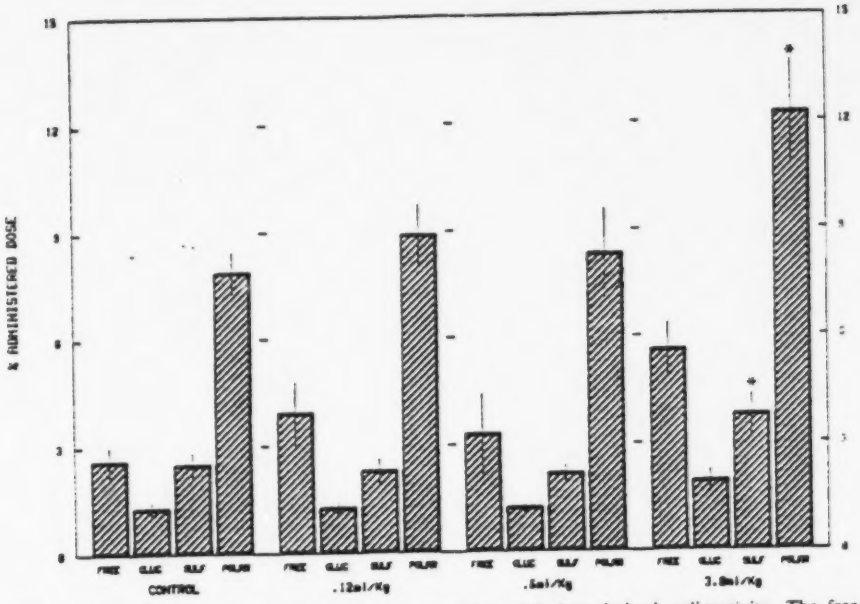

Fig. 1. Effect of ethanol pretreatment on the excretion of lindane-derived radioactivity. The free, glucuronide, sulfate, and polar (mercapturic acid) metabolites were extracted and analyzed as described in METHODS. Each bar is the mean of 6 rats. The vertical lines indicate the SE. *, Significant difference from control.

The effect of ethanol on conjugation reactions tended to be selective. While the metabolism of neither of the glucuronyltransferase enzymes was increased, glutathione-*S*-transferase activity with 1,2-dichloro-4-nitrobenzene as the substrate was increased while that with 1,2-epoxy-3-(*p*-nitrophenoxypropane) was not (Table III).

The in vivo data were in agreement with the in vitro measurements in that ethanol had little effect on most phase I pathways and was selective in its effect on conjugation reactions. For example, while there was no significant increase in total excreted glucuronides (Fig. 1), the excretion of alcohol glucuronides and alcohol sulfates were significantly elevated (Table IV). However, since the oxidation of lindane to the alcohol metabolites was also significantly increased, the proportion of the alcohols excreted as glucuronides and sulfates was calculated (Table V). The proportion of alcohols excreted as glucuronides was significantly higher in rats pretreated with 3 ml/kg ethanol than in any other treatment group, whereas there were no significant differences in the percentage excreted as sulfates. This was interpreted as indicating a selective effect of ethanol on the glucuronidation of the alcohol metabolites. The increased excretion of mercapturic acids was accompanied by a significantly greater amount of radioactivity in the polar urine fraction from rats

TABLE IV

EFFECT OF PRETREATMENT WITH ETHANOL ON PHASE I AND PHASE II REACTIONS IN VIVO

Treatment	Total alcohol excretion[a]	Glucuronidation		Sulfation		Glutathione conjugation
		Alcohol glucuronides[b]	Chlorophenol glucuronides[c]	Alcohol sulfates[d]	Chlorophenol sulfates[e]	Total mercapturic acid conjugates[f]
1. Control	0.72 ± 0.05	0.18 ± 0.02	0.66 ± 0.13	0.32 ± 0.04	1.93 ± 0.32	4.28 ± 0.49
2. Ethanol, 0.12 ml/kg	0.88 ± 0.07	0.22 ± 0.03	0.51 ± 0.14	0.33 ± 0.04	1.48 ± 0.35	5.01 ± 0.49
3. Ethanol, 0.60 ml/kg	0.77 ± 0.17	0.20 ± 0.04	0.69 ± 0.14	0.29 ± 0.05	1.79 ± 0.31	4.69 ± 0.79
4. Ethanol, 3.00 ml/kg	1.75 ± 0.21	0.61 ± 0.10	0.92 ± 0.17	0.58 ± 0.06	2.74 ± 0.58	6.89 ± 1.01
Treatment comparison	4 > All	4 > All	N.S.	4 > All	N.S.	4 > All

Values are means ± SEM from 6 rats. Comparisons were considered significantly different at $P<0.05$, Duncan's Multiple Range Test. N.S., comparisons not significantly different at $P<0.05$.

[a]% Administered lindane excreted as alcohol metabolites per 24 h.

[b]% Administered lindane excreted as alcohol glucuronides per 24 h.

[c]% Administered lindane excreted as chlorophenol glucuronides per 24 h.

[d]% Administered lindane excreted as alcohol sulfates per 24 h.

[e]% Administered lindane excreted as chlorophenol sulfates per 24 h.

[f]% Administered lindane excreted as mercapturic acids per 24 h.

TABLE V

EFFECT OF PRETREATMENT WITH ETHANOL ON PROPORTION OF EXCRETED ALCOHOL GLUCURONIDES AND ALCOHOL SULFATES

Treatment	% Alcohols excreted as glucuronides[a]	% Alcohols excreted as sulfates[b]
1. Control	25.4 ± 2.0	44.7 ± 5.2
2. Ethanol, 0.12 ml/kg	23.1 ± 1.5	37.4 ± 4.0
3. Ethanol, 0.60 ml/kg	26.0 ± 2.2	39.9 ± 3.7
4. Ethanol, 3.00 ml/kg	34.0 ± 2.0	33.8 ± 1.6
Treatment comparison	4 > All	N.S.

Values are means ± SEM from 6 rats. Comparisons were considered significantly different at $P<0.05$, Duncan's Multiple Range Test. N.S., comparisons not significantly different at $P<0.05$.

[a]% Total alcohols excreted as glucuronides.

[b]% Total alcohols excreted as sulfates.

dosed with 3 ml/kg ethanol. Differences between radioactivity of the polar urinary fraction and mercapturic acid content determined by gas-liquid chromatography analysis may indicate the presence of polar metabolites other than mercapturic acids.

DISCUSSION

The in vitro results of the present study suggest a minimal effect for repeated administration of ethanol at low doses despite the reports that induction can occur [1,2]. More importantly the in vivo results measuring the actual effects in an intact animal suggest that at reasonable levels of ethanol administration little interaction is to be expected. Only one phase I pathway, the oxidation of lindane to alcohol metabolites, was significantly altered by ethanol and then only at the high dose. It has been suggested that oxidation of lindane to alcohol metabolites, such as 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol involves the nucleophilic attack of superoxide anion [23]. None of the other phase I pathways examined in vivo or in vitro were significantly altered in a dose related manner. Conjugation reactions were selective. In vivo, glucuronidation of the alcohol metabolites but not the chlorophenols was significantly elevated. Interestingly, the effects of the ethanol on glutathione conjugation reactions appear to be more of a factor than changes in microsomal metabolism.

This comparative study of the usual in vitro methods for measuring drug metabolism with that of lindane metabolism in the intact rat also points out the usefulness of the latter, which assesses multiple phase I and phase II reactions, in answering questions regarding the actual magnitude of change which might be expected to occur under realistic conditions.

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